B11

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/081722 A2

(51) International Patent Classification[7]: **C12P 13/08** // (C12P 13/08, C12R 1:19)

(21) International Application Number: PCT/EP02/02421

(22) International Filing Date: 6 March 2002 (06.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 16 518.8 3 April 2001 (03.04.2001) DE

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors: RIEPING, Mechthild; Mönkebergstrasse 1, 33619 Bielefeld (DE). **HERMANN, Thomas**; Zirkonstrasse 8, 33739 Bielefeld (DE).

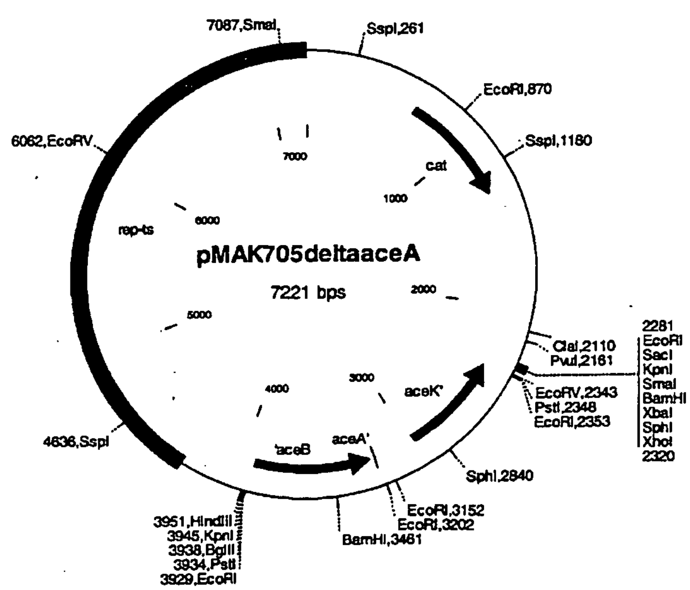
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*
— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE THAT CONTAIN AN ATTENUATED ACEA GENE



(57) Abstract: The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off; b) enrichment of the L-amino acid in the medium or in the cells of the bacteria; and c) isolation of the L-amino acid.

WO 02/081722 A2

Process for the Production of L-Amino Acids using Strains of the Family Enterobacteriaceae that contain an Attenuated aceA Gene

Field of the Invention

The present invention relates to a process for the enzymatic production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae in which the aceA gene is attenuated.

Prior Art

L-amino acids, in particular L-threonine, are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry, and most especially in animal nutrition.

It is known to produce L-amino acids by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. On account of their great importance efforts are constantly being made to improve processes for producing the latter. Process improvements may relate to fermentation technology measures, such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form, for example by ion exchange chromatography, or the intrinsic performance properties of the microorganism itself.

Methods comprising mutagenesis, selection and mutant choice are employed in order to improve the performance properties of these microorganisms. In this way strains are obtained that are resistant to antimetabolites, such as for example the threonine analogue α-amino-β-hydroxyvaleric acid (AHV) or are auxotrophic for regulatorily important metabolites, and that produce L-amino acids such as for example L-threonine.

Methods of recombinant DNA technology have also been used for some years in order to improve strains of the family Enterobacteriaceae producing L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating their effect on production.

Object of the Invention

The object of the invention is to provide new measures for the improved enzymatic production of L-amino acids, in particular L-threonine.

Summary of the Invention

The invention provides a process for the enzymatic production of L-amino acids, in particular L-threonine, using microorganisms of the family Enterobacteriaceae that in particular already produce L-amino acids and in which the nucleotide sequence coding for the aceA gene is attenuated.

Detailed Description of the Invention

Where L-amino acids or amino acids are mentioned hereinafter, this is understood to mean one or more amino acids including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-threonine is particularly preferred.

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by using for example a weak promoter or a gene or allele that codes for a corresponding enzyme with a low activity and/or that inactivates the

corresponding enzyme (protein) or gene, and optionally combining these measures.

By means of these attenuation measures the activity or concentration of the corresponding protein is generally reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein, or the activity or concentration of the protein in the initial microorganism.

The process is characterized in that the following steps are carried out:

a) fermentation of microorganisms of the family Enterobacteriaceae in which the aceA gene is attenuated,

b) enrichment of the corresponding L-amino acid in the medium or in the cells of the microorganisms of the family Enterobacteriaceae, and

c) isolation of the desired L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions thereof remain in the product.

The microorganisms that are the subject of the present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. The microorganisms are members of the family Enterobacteriaceae selected from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. In the case of the genus Escherichia the species Escherichia coli may in particular be mentioned, and in the case of the genus Serratia the species Serratia marcescens may in particular be mentioned.

Suitable strains of the genus Escherichia, in particular those of the species Escherichia coli, that produce in particular L-threonine, include for example:

Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935
Escherichia coli VNIIgenetika MG442
Escherichia coli VNIIgenetika M1
Escherichia coli VNIIgenetika 472T23
Escherichia coli BKIIM B-3996
Escherichia coli kat 13
Escherichia coli KCCM-10132

Suitable strains of the genus Serratia, in particular of the species Serratia marcescens, that produce L-threonine include for example:

Serratia marcescens HNr21
Serratia marcescens TLr156
Serratia marcescens T2000

Strains of the family of Enterobacteriaceae producing L-threonine preferably have, *inter alia*, one or more of the genetic or phenotype features selected from the following group: resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues such as for example valine hydroxamate, resistance to purine analogues such as for example 6-dimethylaminopurine, need for L-methionine, optionally partial and compensable need for L-isoleucine, need for meso-diaminopimelic acid, auxotrophy with regard to threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid,

resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally ability to utilize sucrose, enhancement of the threonine operon, enhancement of homoserine dehydrogenase, I-aspartate kinase I, preferably of the feedback-resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feedback-resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenol pyruvate carboxylase, optionally of the feedback-resistant form, enhancement of phosphoenol pyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

It has now been found that microorganisms of the family Enterobacteriaceae after attenuation, in particular after switching off the aceA gene, produce L-amino acids, in particular L-threonine, in an improved way.

The nucleotide sequences of the Escherichia coli genes belong to the prior art and may also be obtained from the genome sequence of Escherichia coli published by Blattner et al. (Science 277, 1453 - 1462 (1997)).

The aceA gene is described *inter alia* by the following data:

Designation:	Isocitrate lyase
EC-No.:	4.1.3.1
Reference:	Matsuoko and McFadden; Journal of Bacteriology 170, 4528-4536 (1988)
Accession No.:	AE000474

Apart from the described aceA gene, alleles of the gene may be used that result from the degeneracy of the genetic code or from functionally neutral sense mutations, the activity of the protein not being substantially altered.

In order to achieve an attenuation the expression of the gene or the catalytic properties of the enzyme proteins may for example be reduced or switched off. Optionally both measures may be combined.

The gene expression may be reduced by suitable culture conditions, by genetic alteration (mutation) of the signal structures of the gene expression, or also by antisense-RNA techniques. Signal structures of the gene expression are for example repressor genes, activator genes, operators, promoters, attenuators, ribosome-binding sites, the start codon and terminators. The person skilled in the art may find relevant information in, *inter alia*, articles by Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), by Carrier and Keasling (Biotechnology Progress 15, 58-64 (1999), Franch and Gerdes (Current Opinion in Microbiology 3, 159-164 (2000)) and in known textbooks of genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene and Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change or reduction of the catalytic properties of enzyme proteins are known from the prior art. As examples there may be mentioned the work by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences, USA 95, 5511-5515 (1998), Wente and Schachmann (Journal of Biological Chemistry 266, 20833-20839 (1991). Detailed information may be obtained from known textbooks on genetics and molecular biology, such as

for example that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations include transitions, transversions, insertions and deletions. Depending on the action of the amino acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations, which in turn lead to the incorporation of false amino acids or the premature termination of a translation. If as a result of the mutation a stop codon is formed in the coding region, this also leads to a premature termination of the translation. Deletions of several codons typically lead to a complete disruption of the enzyme activity. Details regarding the production of such mutations belong to the prior art and may be obtained from known textbooks on genetics and molecular biology, such as for example the textbook by Knippers ("Molekulare Genetik", 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations in the genes such as for example deletion mutations may be incorporated by gene and/or allele exchange in suitable strains.

A conventional method is the method of gene exchange by means of a conditionally replicating pSC101 derivate pMAK705 described by Hamilton et al. (Journal of Bacteriology 171, 4617 - 4622 (1989)). Other methods described in the prior art, such as for example that of Martinez-Morales et al. (Journal of Bacteriology 181, 7143-7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182, 842-847 (2000)) may likewise be used.

It is also possible to transfer mutations in the respective genes or mutations relating to the expression of the relevant genes, by conjugation or transduction into various strains.

Furthermore for the production of L-amino acids, in particular L-threonine, using strains of the family Enterobacteriaceae it may be advantageous in addition to the attenuation of the aceA gene also to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide-adenine-dinucleotide phosphate.

The term "enhancement" describes in this connection the raising of the intracellular activity of one or more enzymes or proteins in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, using a strong promoter or a gene that codes for a corresponding enzyme or protein having a high activity, and optionally by combining these measures.

By means of the enhancement measures, in particular overexpression, the activity or concentration of the corresponding protein is in general raised by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most up to 1000% or 2000% referred to that of the wild type protein and/or the activity or concentration of the protein in the initial microorganism.

Thus, one or more of the genes selected from the following group may for example by simultaneously enhanced, in particular overexpressed:

- the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),

- the pyc gene coding for pyruvate carboxylase (DE-A-19 831 609),
- the pps gene coding for phosphoenol pyruvate synthase (Molecular and General Genetics 231:332 (1992)),
- the ppc gene coding for phosphoenol pyruvate carboxylase (Gene 31:279-283 (1984)),
- the genes pntA and pntB coding for transhydrogenase (European Journal of Biochemistry 158:647-653 (1986)),
- the gene rhtB imparting homoserine resistance (EP-A-0 994 190),
- the mqo gene coding for malate:quinone oxidoreductase (DE 100 348 33.5),
- the gene rhtC imparting threonine resistance (EP-A-1 013 765), and
- the thrE gene of Corynebacterium glutamicum coding for threonine export (DE 100 264 94.8).

The use of endogenous genes is in general preferred. The term "endogenous genes" or "endogenous nucleotide sequences" is understood to mean the genes or nucleotide sequences present in the population of a species.

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition to the attenuation of the aceA gene also to attenuate, in particular to switch off or reduce the expression of one or more of the genes selected from the following group:

- the tdh gene coding for threonine dehydrogenase (Ravnikar and Somerville, Journal of Bacteriology 169, 4716-4721 (1987)),

- the mdh gene coding for malate dehydrogenase (E.C. 1.1.1.37) (Vogel et al., Archives in Microbiology 149, 36-42 (1987)),

- the gene product of the open reading frame (orf) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),

- the gene product of the open reading frame (orf) ytfP (Accession Number AAC77179 des National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),

- the pckA gene coding for the enzyme phosphoenol pyruvate carboxykinase (Medina et al. (Journal of Bacteriology 172, 7151-7156 (1990)),

- the poxB gene coding for pyruvate oxidase (Grabau and Cronan (Nucleic Acids Research 14 (13), 5449-5460 (1986)),

- the dgsA gene coding for the regulator of the phosphotransferase system (Hosono et al., Bioscience, Biotechnology and Biochemistry 59, 256-251 (1995) and Accession No.: AE000255), and

- the fruR gene coding for the fructose repressor (Jahreis et al., Molecular and General Genetics 226, 332-336 (1991) and Accession No.: AE000118)

Furthermore for the production of L-amino acids, in particular L-threonine, it may be advantageous in addition to the attenuation of the aceA gene also to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention may be cultivated in a batch process (batch cultivation), in a

fed batch process (feed process) or in a repeated fed batch process (repetitive feed process). A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren and periphere Einrichtungen (Vieweg Verlag, Brunswick /Wiesbaden, 1994)).

The culture medium to be used must appropriately satisfy the requirements of the respective strains. Descriptions of culture media of various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

As carbon sources, sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats such as for example soya bean oil, sunflower oil, groundnut oil and coconut oil, fatty acids such as for example palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid, may be used. These substances may be used individually or as a mixture.

As nitrogen source, organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, maize starch water, soya bean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate may be used. The nitrogen sources may be used individually or as a mixture.

As phosphorus source, phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used. The culture medium must furthermore contain salts of metals,

such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Apart from these, suitable precursors may be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single batch or may be metered in in an appropriate manner during the cultivation.

In order to regulate the pH of the culture basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid are used as appropriate. In order to control foam formation antifoaming agents such as for example fatty acid polyglycol esters may be used. In order to maintain the stability of plasmids, suitable selectively acting substances, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as for example air are fed into the culture. The temperature of the culture is normally 25°C to 45°C, and preferably 30°C to 40°C. Cultivation is continued until a maximum amount of L-amino acids (or L-threonine) has been formed. This target is normally achieved within 10 hours to 160 hours.

The L-amino acids may be analyzed by anion exchange chromotography followed by ninhydrin derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention can be used for the enzymatic production of L-amino acids, such as for example L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

A pure culture of the Escherichia coli K-12 strain DH5α/pMAK705 was filed as DSM 13720 on 08 September 2000 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany) according to the Budapest Convention.

The present invention is described in more detail hereinafter with the aid of examples of implementation.

The isolation of plasmid DNA from Escherichia coli as well as all techniques for the restriction, Klenow treatment and alkaline phosphatase treatment are carried out according to Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). The transformation of Escherichia coli is, unless otherwise described, carried out according to Chung et al. (Proceedings of the National Academy of Sciences of the United States of America, USA (1989) 86: 2172-2175).

The incubation temperature in the production of strains and transformants is 37°C. In the gene exchange process according to Hamilton et al., temperatures of 30°C and 44°C are used.

<u>Example 1</u>

Construction of the deletion mutation of the aceA gene.

Parts of the gene regions lying upstream and downstream of the aceA gene are amplified from Escherichia coli K12 using the polymerase chain reaction (PCR) as well as synthetic oligonucleotides. Starting from the nucleotide sequence of the aceBAK operon in E. coli K12 MG1655 DNA (SEQ ID No. 1) the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

aceA'5'-1: 5' - ATGCTTACTCACGCCTGTTG - 3' (SEQ ID No. 3)

aceA'5'-[illegible]: 5' - [illegible] - 3' (SEQ ID No. [illegible])

aceA'3'-1: 5' - CAACAACAACCGTTGCTGAC - 3' (SEQ ID No. 5)

aceA'3'-2: 5' - CAGTTCGTTCGCCACCTGTA - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA used for the PCR is isolated according to the manufacturer's instructions using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A ca. 700 bp large DNA fragment from the region lying upstream of the aceA gene (designated 'aceB) and a ca. 800 bp large DNA fragment from the region lying downstream of the aceA gene (designated aceK') can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Taq DNA polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products are ligated according to the manufacturer's instructions in each case with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, Netherlands) and transformed in the E. coli strain TOP10F'. The selection of plasmid-carrying cells is carried out on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA isolation the vector pCR2.1TOPO'aceB is cleaved with the restriction enzymes EcoRV and SpeI, and the 'aceB fragment after separation in 0.8% agarose gel is isolated using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After the plasmid DNA isolation the vector pCR2.1TOPOaceK' is cleaved with the enzymes Ecl136II and SpeI and ligated with the isolated 'aceB fragment. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar to which 50 µg/ml of ampicillin has been added. After the plasmid DNA isolation, those plasmids in which the mutagenic DNA sequence shown in SEQ ID No. 7 is present in cloned form are detected by control cleavage with the enzymes HindIII and XbaI. One of the plasmids is designated pCR2.1TOPOΔaceA.

Example 2

Construction of the exchange vector pMAK705ΔaceA

The aceBAK allele described in Example 1 is isolated from the vector pCR2.1TOPOΔaceA after restriction with the enzymes HindIII and XbaI and separation in 0.8% agarose gel, and is ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622), that had been digested with the enzymes HindIII and XbaI. The ligation batch is transformed in DH5α and plasmid-carrying cells are selected on LB agar to which 20 µg/ml of chloramphenicol have been added. The successful cloning is detected after plasmid DNA isolation and cleavage with the enzymes BamHI, KpnI, SphI, SpeI and PstI. The resultant exchange vector pMAK705ΔaceA (= pMAK705deltaaceA) is shown in Fig. 1.

Example 3

Site-specific mutagenesis of the aceA gene in the E. coli strain MG442

The E. coli strain MG442 producing L-threonine is described in patent specification US-A- 4,278,765 and is filed as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

For the exchange of the chromosomal aceA gene by the plasmid-coded deletion construct, MG442 is transformed with the plasmid pMAK705ΔaceA. The gene exchange is carried out by the selection process described by Hamilton et al. (1989) Journal of Bacteriology 171, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with the following oligonucleotide primers:

aceA'5'-1: 5' - ATGCTTACTCACGCCTGTTG - 3' (SEQ ID No. 3)

aceA'3'-2: 5' - CAGTTCGTTCGCCACCTGTA - 3' (SEQ ID No. 6)

The resultant strain is designated MG442ΔaceA.

Example 4

Production of L-threonine using the strain MG442ΔaceA

MG442ΔaceA is cultivated on minimal medium having the following composition: 3.5 g/l $Na_2HPO_4 \cdot 2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l glucose and 20 g/l agar. The formation of L-threonine is checked in batch cultures of 10 ml that are contained in 100 ml Erlenmeyer flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose are inoculated and incubated for 16 hours at 37°C and 180 rpm in an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 µl of this preculture are reinoculated in 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4 \cdot 7H_2O$, 0.03 g/l $FeSO_4 \cdot 7H_2O$, 0.018 g/l $MnSO_4 \cdot 1H_2O$, 30 g/l $CaCO_3$ and 20 g/l glucose) and incubated for 48 hours at 37°C. After incubation the optical density (OD) of the culture suspension is measured with an LP2W photometer from the Dr. Lange company (Dusseldorf, Germany) at a measurement wavelength of 660 nm.

The concentration of formed L-threonine is then determined in the sterile-filtered culture supernatant using an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the test is given in Table 1.

Table 1

Strain	OD (660 nm)	L-threonine g/l
MG442	6.0	1.5
MG442ΔaceA	6.2	1.9

Brief Description of the Figure:

- Fig. 1: pMAK705ΔaceA (= pMAK705deltaaceA)

Length data are given as approximate values. The abbreviations and acronyms used have the following meanings:

- cat: chloramphenicol resistance gene
- rep-ts: temperature-sensitive replication region of the plasmid pSC101
- 'aceB: part of the 3` region of the aceB gene
- aceA': ATG start codon of the aceA gene
- aceK': part of the 5` region of the aceK gene

The abbreviations for the restriction enzymes have the following meanings:

- BamHI: restriction endonuclease from Bacillus amyloliquefaciens
- BglII: restriction endonuclease from Bacillus globigii
- ClaI: restriction endonuclease from Caryphanon latum
- EcoRI: restriction endonuclease from Escherichia coli

- EcoRV: restriction endonuclease from Escherichia coli
- HindIII: restriction endonuclease from Haemophilus influenzae
- KpnI: restriction endonuclease from Klebsiella pneumoniae
- PstI: restriction endonuclease from Providencia stuartii
- PvuI: restriction endonuclease from Proteus vulgaris
- SacI: restriction endonuclease from Streptomyces achromogenes
- SalI: restriction endonuclease from Streptomyces albus
- SmaI: restriction endonuclease from Serratia marcescens
- SphI: restriction endonuclease from Streptomyces phaeochromogenes
- SspI: restriction endonuclease from Sphaerotilus species
- XbaI: restriction endonuclease from Xanthomonas badrii
- XhoI: restriction endonuclease from Xanthomonas holcicola

What is Claimed is:

1. Process for the production of L-amino acids, in particular L-threonine, wherein the following steps are carried out:

   a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off,

   b) enrichment of the L-amino acid in the medium or in the cells of the microorganisms, and

   c) isolation of the L-amino acid, in which optionally constituents of the fermentation broth and/or the biomass in its entirety or portions thereof remain in the product.

2. Process according to claim 1, wherein microorganisms are used in which in addition further genes of the biosynethesis pathway of the desired L-amino acid are enhanced.

3. Process according to claim 1, wherein microorganisms are used in which the metabolic pathways that reduce the formation of the desired L-amino acid are at least partially switched off.

4. Process according to claim 1, wherein the expression of the polynucleotide(s) that codes/code for the aceA gene is attenuated, in particular is switched off.

5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide aceA codes are reduced.

6. Process according to claim 1, wherein, for the production of L-amino acids, microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is enhanced, in particular overexpressed:

6.1 the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,

6.2 the pyc gene coding for pyruvate carboxylase,

6.3 the pps gene coding for phosphoenol pyruvate synthase,

6.4 the ppc gene coding for phosphoenol pyruvate carboxylase,

6.5 the genes pntA and pntB coding for transhydrogenase,

6.6 the gene rhtB imparting homoserine resistance,

6.7 the mqo gene coding for malate:quinone oxidoreductase,

6.8 the gene rhtC imparting threonine resistance, and

6.9 the thrE gene coding for threonine export.

7. Process according to claim 1, wherein, for the production of L-amino acids, microorganisms of the family Enterobacteriaceae are fermented in which at the same time one or more of the genes selected from the following group is attenuated, in particular switched off, or the expression is reduced:

7.1 the tdh gene coding for threonine dehydrogenase,

7.2 the mdh gene coding for malate dehydrogenase,

7.3 the gene product of the open reading frame (orf) yjfA,

7.4 the gene product of the open reading frame (orf) ytfP,

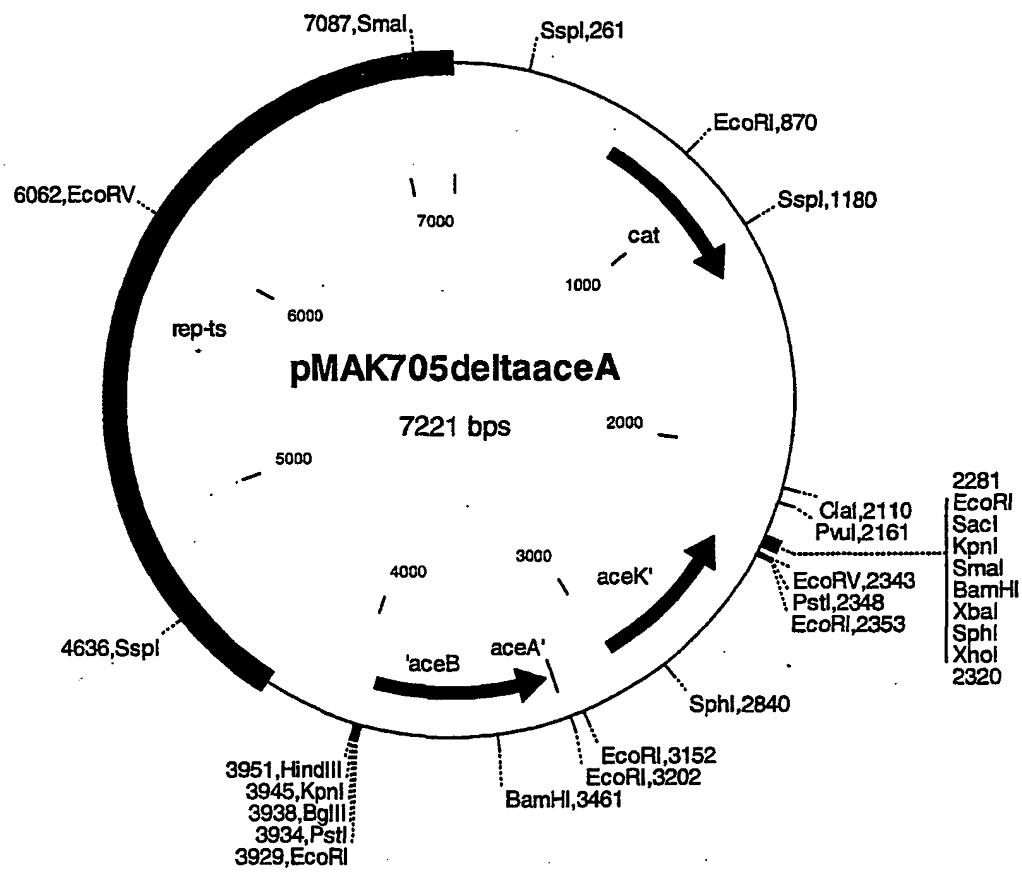
7.5 the pckA gene coding for phosphoenol pyruvate carboxykinase,

7.6 the poxB gene coding for pyruvate oxidase,

7.7 the dgsA gene coding for the regulator of the phosphotransferase system, and

7.8 the fruR gene coding for the fructose repressor.

Fig. 1:


7087,SmaI
SspI,261
EcoRI,870
SspI,1180
6062,EcoRV
7000
cat
1000
6000
rep-ts
pMAK705deltaaceA
7221 bps
2000
5000
ClaI,2110
PvuI,2161
2281
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SphI
XhoI
2320
4000
3000
aceK'
EcoRV,2343
PstI,2348
EcoRI,2353
4636,SspI
'aceB
aceA'
SphI,2840
EcoRI,3152
EcoRI,3202
BamHI,3461
3951,HindIII
3945,KpnI
3938,BglII
3934,PstI
3929,EcoRI

SEQUENCE LISTING

<110> Degussa AG

<120> Process for the production of L-amino acids using strains of the family Enterobacteriaceae containing an attenuated aceA gene

<130> 010131 BT

<160> 7

<170> PatentIn version 3.1

<210> 1
<211> 4855
<212> DNA
<213> Escherichia coli

<220>
<221> CDS
<222> (1632)..(2936)
<223> aceA gene

<400> 1
```
atgactgaac aggcaacaac aaccgatgaa ctggctttca caaggccgta tggcgagcag      60

gagaagcaaa ttcttactgc cgaagcggta gaatttctga ctgagctggt gacgcatttt     120

acgccacaac gcaataaact tctggcagcg cgcattcagc agcagcaaga tattgataac     180

ggaacgttgc ctgattttat ttcggaaaca gcttccattc gcgatgctga ttggaaaatt     240

cgcgggattc ctgcggactt agaagaccgc cgcgtagaga taactggccc ggtagagcgc     300

aagatggtga tcaacgcgct caacgccaat gtgaaagtct ttatggccga tttcgaagat     360

tcactggcac cagactggaa caaagtgatc gacgggcaaa ttaacctgcg tgatgcggtt     420

aacggcacca tcagttacac caatgaagca ggcaaaattt accagctcaa gcccaatcca     480

gcggttttga tttgtcgggt acgcggtctg cacttgccgg aaaaacatgt cacctggcgt     540

ggtgaggcaa tccccggcag cctgtttgat tttgcgctct atttcttcca caactatcag     600

gcactgttgg caaagggcag tggtccctat ttctatctgc cgaaaaccca gtcctggcag     660

gaagcggcct ggtggagcga agtcttcagc tatgcagaag atcgctttaa tctgccgcgc     720

ggcaccatca aggcgacgtt gctgattgaa acgctgcccg ccgtgttcca gatggatgaa     780

atccttcacg cgctgcgtga ccatattgtt ggtctgaact gcggtcgttg ggattacatc     840

ttcagctata tcaaaacgtt gaaaaactat cccgatcgcg tcctgccaga cagacaggca     900

gtgacgatgg ataaaccatt cctgaatgct tactcacgcc tgttgattaa aacctgccat     960

aaacgcggtg cttttgcgat gggcggcatg gcggcgttta ttccgagcaa agatgaagag    1020

cacaataacc aggtgctcaa caaagtaaaa gcggataaat cgctggaagc caataacggt    1080

cacgatggca catggatcgc tcacccaggc cttgcggaca cggcaatggc ggtattcaac    1140
```

```
actgccgatc agctgctggc accttgtgat ggtgaacgca ccgaagaagg tatgcgcgcc   1260

aacattcgcg tggctgtgca gtacatcgaa gcgtggatct ctggcaacgg ctgtgtgccg   1320

atttatggcc tgatggaaga tgcggcgacg gctgaaattt cccgtacctc gatctggcag   1380

tggatccatc atcaaaaaac gttgagcaat ggcaaaccgg tgaccaaagc cttgttccgc   1440

cagatgctgg gcgaagagat gaaagtcatt gccagcgaac tgggcgaaga acgtttctcc   1500

caggggcgtt ttgacgatgc cgcacgcttg atggaacaga tcaccacttc cgatgagtta   1560

attgatttcc tgaccctgcc aggctaccgc ctgttagcgt aaaccaccac ataactatgg   1620

agcatctgca c atg aaa acc cgt aca caa caa att gaa gaa tta cag aaa   1670
             Met Lys Thr Arg Thr Gln Gln Ile Glu Glu Leu Gln Lys
             1               5                   10

gag tgg act caa ccg cgt tgg gaa ggc att act cgc cca tac agt gcg   1718
Glu Trp Thr Gln Pro Arg Trp Glu Gly Ile Thr Arg Pro Tyr Ser Ala
    15                  20                  25

gaa gat gtg gtg aaa tta cgc ggt tca gtc aat cct gaa tgc acg ctg   1766
Glu Asp Val Val Lys Leu Arg Gly Ser Val Asn Pro Glu Cys Thr Leu
30                  35                  40                  45

gcg caa ctg ggc gca gcg aaa atg tgg cgt ctg ctg cac ggt gag tcg   1814
Ala Gln Leu Gly Ala Ala Lys Met Trp Arg Leu Leu His Gly Glu Ser
                50                  55                  60

aaa aaa ggc tac atc aac agc ctc ggc gca ctg act ggc ggt cag gcg   1862
Lys Lys Gly Tyr Ile Asn Ser Leu Gly Ala Leu Thr Gly Gly Gln Ala
            65                  70                  75

ctg caa cag gcg aaa gcg ggt att gaa gca gtc tat ctg tcg gga tgg   1910
Leu Gln Gln Ala Lys Ala Gly Ile Glu Ala Val Tyr Leu Ser Gly Trp
        80                  85                  90

cag gta gcg gcg gac gct aac ctg gcg gcc agc atg tat ccg gat cag   1958
Gln Val Ala Ala Asp Ala Asn Leu Ala Ala Ser Met Tyr Pro Asp Gln
    95                  100                 105

tcg ctc tat ccg gca aac tcg gtg cca gct gtg gtg gag cgg atc aac   2006
Ser Leu Tyr Pro Ala Asn Ser Val Pro Ala Val Val Glu Arg Ile Asn
110                 115                 120                 125

aac acc ttc cgt cgt gcc gat cag atc caa tgg tcc gcg ggc att gag   2054
Asn Thr Phe Arg Arg Ala Asp Gln Ile Gln Trp Ser Ala Gly Ile Glu
                130                 135                 140

ccg ggc gat ccg cgc tat gtc gat tac ttc ctg ccg atc gtt gcc gat   2102
Pro Gly Asp Pro Arg Tyr Val Asp Tyr Phe Leu Pro Ile Val Ala Asp
            145                 150                 155

gcg gaa gcc ggt ttt ggc ggt gtc ctg aat gcc ttt gaa ctg atg aaa   2150
Ala Glu Ala Gly Phe Gly Gly Val Leu Asn Ala Phe Glu Leu Met Lys
        160                 165                 170

gcg atg att gaa gcc ggt gca gcg gca gtt cac ttc gaa gat cag ctg   2198
Ala Met Ile Glu Ala Gly Ala Ala Ala Val His Phe Glu Asp Gln Leu
    175                 180                 185

gcg tca gtg aag aaa tgc ggt cac atg ggc ggc aaa gtt tta gtg cca   2246
Ala Ser Val Lys Lys Cys Gly His Met Gly Gly Lys Val Leu Val Pro
190                 195                 200                 205
```

act cag gaa gct att cag aaa ctg gtc gcg gcg cgt ctg gca gct gac 2294
Thr Gln Glu Ala Ile Gln Lys Leu Val Ala Ala Arg Leu Ala Ala Asp
210 215 220

gtg acg ggc gtt cca acc ctg ctg gtt gcc cgt acc gat gct gat gcg 2342
Val Thr Gly Val Pro Thr Leu Leu Val Ala Arg Thr Asp Ala Asp Ala
225 230 235

gcg gat ctg atc acc tcc gat tgc gac ccg tat gac agc gaa ttt att 2390
Ala Asp Leu Ile Thr Ser Asp Cys Asp Pro Tyr Asp Ser Glu Phe Ile
240 245 250

acc ggc gag cgt acc agt gaa ggc ttc ttc cgt act cat gcg ggc att 2438
Thr Gly Glu Arg Thr Ser Glu Gly Phe Phe Arg Thr His Ala Gly Ile
255 260 265

gag caa gcg atc agc cgt ggc ctg gcg tat gcg cca tat gct gac ctg 2486
Glu Gln Ala Ile Ser Arg Gly Leu Ala Tyr Ala Pro Tyr Ala Asp Leu
270 275 280 285

gtc tgg tgt gaa acc tcc acg ccg gat ctg gaa ctg gcg cgt cgc ttt 2534
Val Trp Cys Glu Thr Ser Thr Pro Asp Leu Glu Leu Ala Arg Arg Phe
290 295 300

gca caa gct atc cac gcg aaa tat ccg ggc aaa ctg ctg gct tat aac 2582
Ala Gln Ala Ile His Ala Lys Tyr Pro Gly Lys Leu Leu Ala Tyr Asn
305 310 315

tgc tcg ccg tcg ttc aac tgg cag aaa aac ctc gac gac aaa act att 2630
Cys Ser Pro Ser Phe Asn Trp Gln Lys Asn Leu Asp Asp Lys Thr Ile
320 325 330

gcc agc ttc cag cag cag ctg tcg gat atg ggc tac aag ttc cag ttc 2678
Ala Ser Phe Gln Gln Gln Leu Ser Asp Met Gly Tyr Lys Phe Gln Phe
335 340 345

atc acc ctg gca ggt atc cac agc atg tgg ttc aac atg ttt gac ctg 2726
Ile Thr Leu Ala Gly Ile His Ser Met Trp Phe Asn Met Phe Asp Leu
350 355 360 365

gca aac gcc tat gcc cag ggc gag ggt atg aag cac tac gtt gag aaa 2774
Ala Asn Ala Tyr Ala Gln Gly Glu Gly Met Lys His Tyr Val Glu Lys
370 375 380

gtg cag cag ccg gaa ttt gcc gcc gcg aaa gat ggc tat acc ttc gta 2822
Val Gln Gln Pro Glu Phe Ala Ala Ala Lys Asp Gly Tyr Thr Phe Val
385 390 395

tct cac cag cag gaa gtg ggt aca ggt tac ttc gat aaa gtg acg act 2870
Ser His Gln Gln Glu Val Gly Thr Gly Tyr Phe Asp Lys Val Thr Thr
400 405 410

att att cag ggc ggc acg tct tca gtc acc gcg ctg acc ggc tcc act 2918
Ile Ile Gln Gly Gly Thr Ser Ser Val Thr Ala Leu Thr Gly Ser Thr
415 420 425

gaa gaa tcg cag ttc taa gcaacaacaa ccgttgctga ctgtaggccg 2966
Glu Glu Ser Gln Phe
430

gataaggcgt tcacgccgca tccggcaatc ggtgcacgat gcctgatgcg acgcttgcgc 3026

gtcttatcat gcctacagcc gttgccgaac gtaggctgga taaggcgttt acgccgcatc 3086

[illegible]

ctcaaaccat tttgcaaggc ttcgatgctc agtatggtcg attcctcgaa gtgacctccg 3206
gtgcgcagca gcgtttcgaa caggccgact ggcatgctgt ccagcaggcg atgaaaaacc 3266
gtatccatct ttacgatcat cacgttggtc tggtcgtgga gcaactgcgc tgcattacta 3326
acggccaaag tacggacgcg gcatttttac tacgtgttaa agagcattac acccggctgt 3386
tgccggatta cccgcgcttc gagattgcgg agagcttttt taactccgtg tactgtcggt 3446
tatttgacca ccgctcgctt actcccgagc ggctttt0tat ctttagctct cagccagagc 3506
gccgctttcg taccattccc cgcccgctgg cgaaagactt tcaccccgat cacggctggg 3566
aatctctact gatgcgcgtt atcagcgacc taccgctgcg cctgcgctgg cagaataaaa 3626
gccgtgacat ccattacatt attcgccatc tgacggaaac gctggggaca gacaacctcg 3686
cggaaagtca tttacaggtg gcgaacgaac tgttttaccg caataaagcc gcctggctgg 3746
taggcaaact gatcacacct tccggcacat tgccattttt gctgccgatc caccagacgg 3806
acgacggcga gttatttatt gatacctgcc tgacgacgac cgccgaagcg agcattgttt 3866
ttggctttgc gcgttcttat tttatggttt atgcgccgct gcccgcagcg ctggtcgagt 3926
ggctacggga aattctgcca ggtaaaacca ccgctgaatt gtatatggct atcggctgcc 3986
agaagcacgc caaaaccgaa agctaccgcg aatatctcgt ttatctacag ggctgtaatg 4046
agcagttcat tgaagcgccg ggtattcgtg gaatggtgat gttggtgttt acgctgccgg 4106
gctttgatcg ggtattcaaa gtcatcaaag acaggttcgc gccgcagaaa gagatgtctg 4166
ccgctcacgt tcgtgcctgc tatcaactgg tgaaagagca cgatcgcgtg ggccgaatgg 4226
cggacaccca ggagtttgaa aactttgtgc tggagaagcg gcatatttcc ccggcattaa 4286
tggaattact gcttcaggaa gcagcggaaa aaatcaccga tctcggcgaa caaattgtga 4346
ttcgccatct ttatattgag cggcggatgg tgccgctcaa tatctggctg gaacaagtgg 4406
aaggtcagca gttgcgcgac gccattgaag aatacggtaa cgctattcgc cagcttgccg 4466
ctgctaacat tttccctggc gacatgctgt ttaaaaactt cggtgtcacc cgtcacgggc 4526
gtgtggtttt ttatgattac gatgaaattt gctacatgac ggaagtgaat ttccgcgaca 4586
tcccgccgcc gcgctatccg gaagacgaac ttgccagcga accgtggtac agcgtctcgc 4646
cgggcgatgt tttcccggaa gagtttcgcc actggctatg cgccgacccg cgtattggtc 4706
cgctgtttga agagatgcac gccgacctgt tccgcgctga ttactggcgc gcactacaaa 4766
accgcatacg tgaagggcat gtggaagatg tttatgcgta tcggcgcagg caaagattta 4826
gcgtacggta tggggagatg cttttttga 4855

<210> 2
<211> 434
<212> PRT
<213> Escherichia coli

```
<400>  2
Met Lys Thr Arg Thr Gln Gln Ile Glu Glu Leu Gln Lys Glu Trp Thr
1               5                   10                  15

Gln Pro Arg Trp Glu Gly Ile Thr Arg Pro Tyr Ser Ala Glu Asp Val
            20                  25                  30

Val Lys Leu Arg Gly Ser Val Asn Pro Glu Cys Thr Leu Ala Gln Leu
        35                  40                  45

Gly Ala Ala Lys Met Trp Arg Leu Leu His Gly Glu Ser Lys Lys Gly
    50                  55                  60

Tyr Ile Asn Ser Leu Gly Ala Leu Thr Gly Gly Gln Ala Leu Gln Gln
65                  70                  75                  80

Ala Lys Ala Gly Ile Glu Ala Val Tyr Leu Ser Gly Trp Gln Val Ala
                85                  90                  95

Ala Asp Ala Asn Leu Ala Ala Ser Met Tyr Pro Asp Gln Ser Leu Tyr
            100                 105                 110

Pro Ala Asn Ser Val Pro Ala Val Val Glu Arg Ile Asn Asn Thr Phe
        115                 120                 125

Arg Arg Ala Asp Gln Ile Gln Trp Ser Ala Gly Ile Glu Pro Gly Asp
    130                 135                 140

Pro Arg Tyr Val Asp Tyr Phe Leu Pro Ile Val Ala Asp Ala Glu Ala
145                 150                 155                 160

Gly Phe Gly Gly Val Leu Asn Ala Phe Glu Leu Met Lys Ala Met Ile
                165                 170                 175

Glu Ala Gly Ala Ala Ala Val His Phe Glu Asp Gln Leu Ala Ser Val
            180                 185                 190

Lys Lys Cys Gly His Met Gly Gly Lys Val Leu Val Pro Thr Gln Glu
        195                 200                 205

Ala Ile Gln Lys Leu Val Ala Ala Arg Leu Ala Ala Asp Val Thr Gly
    210                 215                 220

Val Pro Thr Leu Leu Val Ala Arg Thr Asp Ala Asp Ala Ala Asp Leu
225                 230                 235                 240

Ile Thr Ser Asp Cys Asp Pro Tyr Asp Ser Glu Phe Ile Thr Gly Glu
                245                 250                 255

Arg Thr Ser Glu Gly Phe Phe Arg Thr His Ala Gly Ile Glu Gln Ala
            260                 265                 270

Ile Ser Arg Gly Leu Ala Tyr Ala Pro Tyr Ala Asp Leu Val Trp Cys
        275                 280                 285

Glu Thr Ser Thr Pro Asp Leu Glu Leu Ala Arg Arg Phe Ala Gln Ala
    290                 295                 300

Ile His Ala Lys Tyr Pro Gly Lys Leu Leu Ala Tyr Asn Cys Ser Pro
305                 310                 315                 320

Ser Phe Asn Trp Gln Lys Asn Leu Asp Asp Lys Thr Ile Ala Ser Phe
                325                 330                 335

Gln Gln Gln Leu Val Asp Asn Gly Tyr Lys Phe Gln Phe Ile Gln Leu
```

```
            340                 345                 350

Ala Gly Ile His Ser Met Trp Phe Asn Met Phe Asp Leu Ala Asn Ala
        355                 360                 365

Tyr Ala Gln Gly Glu Gly Met Lys His Tyr Val Glu Lys Val Gln Gln
    370                 375                 380

Pro Glu Phe Ala Ala Ala Lys Asp Gly Tyr Thr Phe Val Ser His Gln
385                 390                 395                 400

Gln Glu Val Gly Thr Gly Tyr Phe Asp Lys Val Thr Thr Ile Ile Gln
                405                 410                 415

Gly Gly Thr Ser Ser Val Thr Ala Leu Thr Gly Ser Thr Glu Glu Ser
            420                 425                 430

Gln Phe


<210>  3
<211>  20
<212>  DNA
<213>  Artificial sequence

<220>
<221>  Primer
<222>  (1)..(20)
<223>  aceA `5`-1

<400>  3
atgcttactc acgcctgttg                                                20

<210>  4
<211>  20
<212>  DNA
<213>  Artificial sequence

<220>
<221>  Primer
<222>  (1)..(20)
<223>  aceA `5`-2

<400>  4
catgtgcaga tgctccatag                                                20


<210>  5
<211>  20
<212>  DNA
<213>  Artificial sequence

<220>
<221>  Primer
<222>  (1)..(20)
<223>  aceA `3`-1

<400>  5
caacaacaac cgttgctgac                                                20

<210>  6
<211>  20
<212>  DNA
<213>  Artificial sequence
```

<220>
<221> Primer
<222> (1)..(20)
<223> aceA `3`-2

<400> 6
cagttcgttc gccacctgta 20

<210> 7
<211> 1643
<212> DNA
<213> Escherichia coli

<220>
<221> misc_feature
<222> (1)..(33)
<223> Technical DNA/ remainder polylinker sequence

<220>
<221> misc_feature
<222> (34)..(742)
<223> Part of the 3` region of the aceB gene, intergenic region, ATG start codon of the aceA gene

<220>
<221> misc_feature
<222> (743)..(810)
<223> Technical DNA/ remainder polylinker sequence

<220>
<221> misc_feature
<222> (811)..(1591)
<223> Part of the 5' region of the aceK gene

<220>
<221> misc_feature
<222> (1592)..(1643)
<223> Technical DNA/ remainder polylinker sequence

<400> 7
agcttggtac cgagatctgc agaattcgcc cttatgctta ctcacgcctg ttgattaaaa 60

cctgccataa acgcggtgct tttgcgatgg gcggcatggc ggcgtttatt ccgagcaaag 120

atgaagagca caataaccag gtgctcaaca aagtaaaagc ggataaatcg ctggaagcca 180

ataacggtca cgatggcaca tggatcgctc acccaggcct tgcggacacg gcaatggcgg 240

tattcaacga cattctcggc tcccgtaaaa atcagcttga agtgatgcgc gaacaagacg 300

cgccgattac tgccgatcag ctgctggcac cttgtgatgg tgaacgcacc gaagaaggta 360

tgcgcgccaa cattcgcgtg gctgtgcagt acatcgaagc gtggatctct ggcaacggct 420

gtgtgccgat ttatggcctg atggaagatg cggcgacggc tgaaatttcc cgtacctcga 480

tctggcagtg gatccatcat caaaaaacgt tgagcaatgg caaaccggtg accaaagcct 540

tgttccgcca gatgctgggc gaagagatga aagtcattgc cagcgaactg ggcgaagaac 600

gtttctccca ggggcgtttt gacgatgccg cacgcttgat ggaacagatc accacttccg 660

[illegible] [illegible] [illegible] [illegible] gttagcgtaa accaccacat 720

```
aactatggag catctgcaca tgaagggcga attccagcac actggcggcc gttactagta    780
acggccgcca gtgtgctgga attcgccctt caacaacaac cgttgctgac tgtaggccgg    840
ataaggcgtt cacgccgcat ccggcaatcg gtgcacgatg cctgatgcga cgcttgcgcg    900
tcttatcatg cctacagccg ttgccgaacg taggctggat aaggcgttta cgccgcatcc    960
ggcaattctc tgctcctgat gagggcgcta aatgccgcgt ggcctggaat tattgattgc   1020
tcaaaccatt ttgcaaggct tcgatgctca gtatggtcga ttcctcgaag tgacctccgg   1080
tgcgcagcag cgtttcgaac aggccgactg gcatgctgtc cagcaggcga tgaaaaaccg   1140
tatccatctt tacgatcatc acgttggtct ggtcgtggag caactgcgct gcattactaa   1200
cggccaaagt acggacgcgg catttttact acgtgttaaa gagcattaca cccggctgtt   1260
gccggattac ccgcgcttcg agattgcgga gagctttttt aactccgtgt actgtcggtt   1320
atttgaccac cgctcgctta ctcccgagcg gctttttatc tttagctctc agccagagcg   1380
ccgctttcgt accattcccc gcccgctggc gaaagacttt caccccgatc acggctggga   1440
atctctactg atgcgcgtta tcagcgacct accgctgcgc ctgcgctggc agaataaaag   1500
ccgtgacatc cattacatta ttcgccatct gacggaaacg ctggggacag acaacctcgc   1560
ggaaagtcat ttacaggtgg cgaacgaact gaagggcgaa ttctgcagat atccatcaca   1620
ctggcggccg ctcgagcatg cat                                           1643
```

PCT 010131 BT

Original (for **SUBMISSION**) - printed on 05.03.2002 09:26:15 AM

0-1	**Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)**	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.01.2002)
0-2	**International Application No.**	
0-3	**Applicant's or agent's file reference**	010131 BT

1	**The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:**	
1-1	page	13
1-2	line	1-5
1-3	**Identification of Deposit**	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	08 September 2000 (08.09.2000)
1-3-4	Accession Number	DSMZ 13720
1-4	**Additional Indications**	NONE
1-5	**Designated States for Which Indications are Made**	all designated States
1-6	**Separate Furnishing of Indications** These indications will be submitted to the International Bureau later	NONE

FOR RECEIVING OFFICE USE ONLY

0-4	**This form was received with the international application:** (yes or no)	YES
0-4-1	Authorized officer	B. GATINET (0)70/3402181

FOR INTERNATIONAL BUREAU USE ONLY

0-5	**This form was received by the international Bureau on:**	
0-5-1	Authorized officer	

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa-Hüls AG
Kantstr. 2
33790 Halle

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: DH5α/pMAK705	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: (X) a scientific description (X) a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2000-09-08 (Date of the original deposit)[1].	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Dagmar Fritze Date: 2000-09-12

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 0196

PCT/EP02/02421

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa-Hüls AG
Kantstr. 2
33790 Halle

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa-Hüls AG Kantstr. 2 Address: 33790 Halle	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13720 Date of the deposit or the transfer[1]: 2000-09-08

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2000-09-08
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Dagmar Fritze Date: 2000-09-12

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

Form [illegible] (sole page) [illegible]

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 October 2002 (17.10.2002)

PCT

(10) International Publication Number
WO 02/081722 A3

(51) International Patent Classification[7]: **C12P 13/08**, 13/04 // (C12P 13/08, C12R 1:19)

(21) International Application Number: PCT/EP02/02421

(22) International Filing Date: 6 March 2002 (06.03.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
101 16 518.8 3 April 2001 (03.04.2001) DE

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors: RIEPING, Mechthild; Mönkebergstrasse 1, 33619 Bielefeld (DE). **HERMANN, Thomas**; Zirkonstrasse 8, 33739 Bielefeld (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

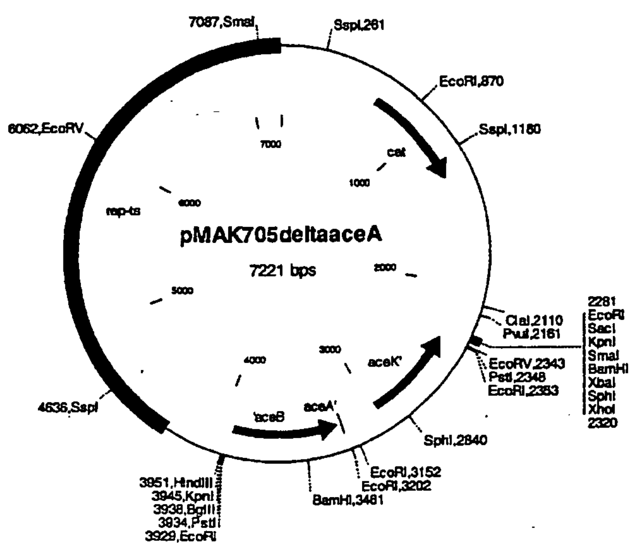
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

(88) Date of publication of the international search report:
30 October 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE THAT CONTAIN AN ATTENUATED ACEA GENE



(57) **Abstract:** The invention relates to a process for the production of L-amino acids, in particular L-threonine, in which the following steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which the aceA gene or nucleotide sequences coding therefor are attenuated, in particular are switched off; b) enrichment of the L-amino acid in the medium or in the cells of the bacteria; and c) isolation of the L-amino acid.

WO 02/081722 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/02421

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/08 C12P13/04 //(C12P13/08,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4 368 266 A (TOSAKA OSAMU ET AL) 11 January 1983 (1983-01-11) column 1, line 39 -column 2, line 8 examples 1-3 tables 3-5 claim 1	1-7
Y	EP 0 952 221 A (AJINOMOTO KK) 27 October 1999 (1999-10-27) page 2, line 24 -page 3, line 2 page 6, line 42 -page 7, line 6 claim 1	1-7
Y	EP 0 955 368 A (AJINOMOTO KK) 10 November 1999 (1999-11-10) page 2, line 22-56 page 6, line 9-31 claim 6	1-7

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 2003	02/06/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van de Kamp, M

Intern ial Application No
PCT/EP 02/02421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MATSUOKA M ET AL.: "Isolation, hyperexpression, and sequencing of the aceA gene encoding isocitrate lyase in Escherichia coli" JOURNAL OF BACTERIOLOGY, vol. 170, no. 10, 1988, pages 4528-4536, XP008016923 ISSN: 0021-9193 cited in the application abstract	1-7
A	MICHAL G: "Biochemical pathways: an atlas of biochemistry and molecular biology" 1999 , JOHN WILEY & SONS INC. AND SPEKTRUM AKADEMISCHER VERLAG , NEW YORK - HEIDELBERG XP002240819 ISBN: 0-471-33130-9 figures 3.8-2, 3.9-1 figures 4.2-1, 4.5-1 and 4.5-2	1-7
A	KRAEMER R: "Genetic and physiological approaches for the production of amino acids" JOURNAL OF BIOTECHNOLOGY, vol. 45, no. 1, 1996, pages 1-21, XP002178648 ISSN: 0168-1656 the whole document	1-7
A	US 4 278 765 A (DEBABOV VLADIMIR G ET AL) 14 July 1981 (1981-07-14) cited in the application the whole document	1-7
A	EP 0 643 135 A (AJINOMOTO KK) 15 March 1995 (1995-03-15) the whole document	1-7
A	EP 0 237 819 A (KYOWA HAKKO KOGYO KK) 23 September 1987 (1987-09-23) the whole document	1-7
A	DATABASE WPI Section Ch, Week 199148 Derwent Publications Ltd., London, GB; Class B05, AN 1991-351136 XP002241222 & JP 03 236786 A (KYOWA HAKKO KOGYO KK), 22 October 1991 (1991-10-22) abstract	1-7

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/02421

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 53035 A (ALTMAN ELLIOT ;GOKARN RAVI R (US); EITEMAN MARK A (US); UNIV GEORG) 21 October 1999 (1999-10-21) page 5, line 20-24 examples 4,7,9,10 claims 41,49 figures 1,4	1-7
A	JETTEN M S M ET AL.: "Recent advances in the physiology and genetics of amino acid-producing bacteria." CRC CRITICAL REVIEWS IN BIOTECHNOLOGY, vol. 15, no. 1, 1995, pages 73-103, XP000613291 ISSN: 0738-8551 figure 1 page 83, right-hand column, line 1-36 page 90, left-hand column, line 1 -page 92, left-hand column, line 17	1-7
A	CHUNG T ET AL.: "Glyoxylate bypass operon of Escherichia coli: cloning and determination of the functional map." JOURNAL OF BACTERIOLOGY, vol. 170, no. 1, January 1988 (1988-01), pages 386-392, XP008015355 ISSN: 0021-9193 abstract	1-7
A	SAWERS G: "The anaerobic degradation of L-serine and L-threonine in enterobacteria: networks of pathways and regulatory signals" ARCHIVES OF MICROBIOLOGY, vol. 171, no. 1, 1998, pages 1-5, XP002953871 ISSN: 0302-8933 the whole document	1-7
E	WO 02 081698 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 21 -page 10, line 22 claim 7	1-7
E	WO 02 081721 A (DEGUSSA) 17 October 2002 (2002-10-17) the whole document page 9, line 24 -page 10, line 25 claim 7	1-7

INTERNATIONAL SEARCH REPORT

....mation on patent family members

Intern al Application No
PCT/EP 02/02421

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 4368266	A	11-01-1983	JP	1426831	C	25-02-1988
			JP	57018990	A	30-01-1982
			JP	62036677	B	07-08-1987
			JP	1426801	C	25-02-1988
			JP	56092794	A	27-07-1981
			JP	62036675	B	07-08-1987
			FR	2472610	A1	03-07-1981
EP 0952221	A	27-10-1999	AU	756507	B2	16-01-2003
			AU	2122399	A	30-09-1999
			BR	9901173	A	28-03-2000
			CN	1233660	A	03-11-1999
			EP	0952221	A2	27-10-1999
			JP	2000189169	A	11-07-2000
			PL	332072	A1	27-09-1999
			US	6331419	B1	18-12-2001
			US	2001019836	A1	06-09-2001
EP 0955368	A	10-11-1999	AU	746542	B2	02-05-2002
			AU	2122499	A	30-09-1999
			BR	9901174	A	28-03-2000
			CN	1233661	A	03-11-1999
			EP	0955368	A2	10-11-1999
			JP	2000106869	A	18-04-2000
			PL	332071	A1	27-09-1999
			RU	2188236	C2	27-08-2002
			US	6197559	B1	06-03-2001
			US	2002004231	A1	10-01-2002
US 4278765	A	14-07-1981	SU	875663	A1	15-09-1982
			HU	190999	B	28-12-1986
EP 0643135	A	15-03-1995	AT	203769	T	15-08-2001
			CZ	9401658	A3	15-12-1994
			DE	69330518	D1	06-09-2001
			DE	69330518	T2	08-05-2002
			DK	643135	T3	15-10-2001
			EP	0643135	A1	15-03-1995
			JP	3331472	B2	07-10-2002
			SK	81994	A3	10-05-1995
			US	5661012	A	26-08-1997
			EP	1020526	A2	19-07-2000
			ES	2158867	T3	16-09-2001
			WO	9411517	A1	26-05-1994
			RU	2113484	C1	20-06-1998
EP 0237819	A	23-09-1987	DE	3788583	D1	10-02-1994
			DE	3788583	T2	19-05-1994
			EP	0237819	A2	23-09-1987
			JP	2574786	B2	22-01-1997
			JP	63273487	A	10-11-1988
			KR	9108634	B1	19-10-1991
			US	5017483	A	21-05-1991
JP 3236786	A	22-10-1991	JP	2877414	B2	31-03-1999
WO 9953035	A	21-10-1999	AU	3555999	A	01-11-1999
			BR	9909615	A	12-12-2000

Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/02421

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9953035	A		CA	2325598	A1	21-10-1999
			EP	1073722	A1	07-02-2001
			JP	2002511250	T	16-04-2002
			WO	9953035	A1	21-10-1999
			US	2003087381	A1	08-05-2003
			US	6455284	B1	24-09-2002
WO 02081698	A	17-10-2002	DE	10116518	A1	17-10-2002
			WO	02081721	A2	17-10-2002
			WO	02081698	A2	17-10-2002
			WO	02081722	A2	17-10-2002
			US	2003054503	A1	20-03-2003
			US	2003059903	A1	27-03-2003
WO 02081721	A	17-10-2002	DE	10116518	A1	17-10-2002
			WO	02081721	A2	17-10-2002
			WO	02081698	A2	17-10-2002
			WO	02081722	A2	17-10-2002
			US	2003054503	A1	20-03-2003
			US	2003059903	A1	27-03-2003